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Morphogenesis, Adhesive Properties, and Antifungal Resistance Depend on the Pmt6 Protein Mannosyltransferase in the Fungal Pathogen *Candida albicans*

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Protein mannosyltransferases (Pmt proteins) initiate O glycosylation of secreted proteins in fungi. We have characterized *PMT6*, which encodes the second Pmt protein of the fungal pathogen *Candida albicans*. The residues of Pmt6p are 21 and 42% identical to those of *C. albicans* Pmt1p and *S. cerevisiae* Pmt6p, respectively. Mutants lacking one or two *PMT6* alleles grow normally and contain normal Pmt enzymatic activities in cell extracts but show phenotypes including a partial block of hyphal formation (dimorphism) and a supersensitivity to hygromycin B. The morphogenetic defect can be suppressed by overproduction of known components of signaling pathways, including Cek1p, Cph1p, Tpk2p, and Efg1p, suggesting a specific Pmt6p target protein upstream of these components. Mutants lacking both *PMT1* and *PMT6* are viable and show *pmt1* mutant phenotypes and an additional sensitivity to the iron chelator ethylenediamine-di(*o*-hydroxyphenylacetic acid). The lack of Pmt6p significantly reduces adherence to endothelial cells and overall virulence in a mouse model of systemic infection. The results suggest that Pmt6p regulates a more narrow subclass of proteins in *C. albicans* than Pmt1p, including secreted proteins responsible for morphogenesis and antifungal sensitivities.

Secreted proteins in fungi can get modified by the attachment of short glycosyl chains consisting of one to seven mannoses to serine or threonine residues (reviewed in reference 38). The first mannosylation step in O glycosylation occurs in the endoplasmic reticulum, presumably cotranslationally, and is mediated by protein mannosyltransferases (Pmt proteins). In the yeast *Saccharomyces cerevisiae* seven *PMT* genes are known (20, 24, 28, 37); their paralogous gene products, by their degree of homology, can be grouped in at least two subclasses consisting of either the Pmt1 and Pmt5 proteins or the Pmt2, Pmt3, and Pmt6 proteins (10). We recently isolated and characterized the *PMT1* gene of the important human fungal pathogen *Candida albicans* (41). Pmt homologues in *Drosophila melanogaster* (25) and humans (21) have also been described, and Pmt homologues deduced from "expressed sequence tags" occur in nematodes, plants, and mammals, although their enzymatic functions as Pmt proteins have not yet been demonstrated. In *C. albicans*, *O*-glycosyl chains initiated by Pmt proteins are extended further by mannosyltransferases including Mnt1p (3).

Although the molecular details of target protein recognition by Pmt proteins are unknown, it appears that Pmt proteins can have a preference for certain glycosylation targets. Thus, the lack of Pmt1 and Pmt2 proteins in *S. cerevisiae* mutants affects O glycosylation of a set of secreted proteins overlapping with, but different from, the set affected in mutants lacking Pmt4p (16), and certain cell wall proteins are affected differently by mutations in *PMT* genes (28). Recently, the Axl2p protein, involved in axial budding, was recognized as a specific target of mannosylation by Pmt4p (29). Unknown O-glycosylated proteins are needed for general cell viability, since *S. cerevisiae pmt1 pmt2* double mutants show reduced growth and some triple *pmt* mutants are not viable (15). Likewise, the lack of both *pmt1* alleles in *C. albicans* negatively affects growth (41). On the other hand, specific phenotypes have been observed in *pmt* mutants. *S. cerevisiae pmt1* mutants are partially resistant to K1 killer factor (24) and are unable to grow anaerobically in minimal medium (2). *C. albicans pmt1/pmt1* homozygous and *PMT1/pmt1* heterozygous strains showed an increased sensitivity to aminoglycoside antibiotics and a defect in hyphal morphogenesis (41). A cellular differentiation defect was also observed in *Drosophila* strains lacking the PMT gene homologue *rotated abdomen* (25).

Here we describe a second *PMT* gene of *C. albicans*, *PMT6*, which encodes a Pmt protein highly homologous to *S. cerevisiae* Pmt6p and thus is a representative of the second subgroup of *S. cerevisiae* proteins. We demonstrate that deletion of *PMT6* does not affect growth in general but rather generates specific phenotypes, such as antifungal supersensitivity and defective filamentation. Suppression experiments strongly suggest that O glycosylation by Pmt6p affects a specific component upstream of known signaling cascades, triggering morphogenesis. Defects in properties of adhesion to target cells and the reduced virulence of *pmt6* mutants demonstrate the importance of Pmt6p for cellular differentiation and virulence of *C. albicans*.

MATERIALS AND METHODS

Strains and media. The *C. albicans* strains and the plasmids are listed in Table 1. *C. albicans* strains CAI4 (11) and CAP1-3121 (41) were used for transformations and gene disruptions. Strains were grown in yeast extract-peptone-dextrose (YPD) or SD medium (34), which for Ura$^-$ strains was supplemented with 20 μg of uridine/ml. Transformations were performed using the spheroplast method

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TABLE 1. Strains and plasmids

C. albicans strain(s) or plasmid(s)	Genotype or description	Reference or source
Strains		
SC5314	Prototrophic	11
CAI4	*ura3*Δ::*imm34*/*ura3*Δ::*imm34*	11
CAP1-3	As CAI4, but *PMT1*/*pmt1*Δ::*hisG-URA3-hisG*	41
CAP1-312	As CAI4, but *pmt1*Δ::*hisG-URA3-hisG*/*pmt1*Δ::*hisG*	41
CAP1-3121	As CAI4, but *pmt1*Δ::*hisG*/*pmt1*Δ::*hisG*	41
SS4	CAI8 derivative; *efg1*::ADE2/(*URA3-PCK1p*)::*EFG1*	36
HLC67	*efg1*/*efg1* derivative of CAI4	23
CAP2-1, CAP2-2, CAP2-3, CAP2-4	As CAI4, but *PMT6*/*pmt6*Δ::*hisG-URA3-hisG*	This work
CAP2-23	As CAI4, but *PMT6*/*pmt6*Δ::*hisG*	This work
CAP2-234, CAP2-239	As CAI4, but *pmt6*Δ::hisG/*pmt6*Δ::*hisG-URA3-hisG*	This work
CAP2-2341, CAP2-2391	As CAI4, but *pmt6*Δ::*hisG*/*pmt6*Δ::*hisG*	This work
CPP1	As CAP1-3121, but *PMT6*/*pmt6*Δ::*hisG-URA3-hisG*	This work
CPP11	As CAP1-3121, but *PMT6*/*pmt6*Δ::*hisG*	This work
CPP112, CPP117	As CAP1-3121, but *pmt6*Δ::*hisG*/*pmt6*Δ::*hisG-URA3-hisG*	This work
CPP1123, CPP1171	As CAP1-3121, but *pmt6*Δ::*hisG*/*pmt6*Δ::*hisG*	This work
Plasmids (transformation vectors)		
pRC18	*URA3*-marked *CaARS* vector	36
pRC2312	*URA3*-marked *CaARS* vector	4
pLJ19 (p*CPH1*)	*CPH1* under control of the *ADH1* promoter	7
pCaTPK2 (p*TPK2*)	*URA3*-marked *CaARS* vectors containing *TPK2*	35
pCCa4 (p*CEK1*)	*URA3*-marked *CaARS* vectors containing *CEK1*	7
pBI-HAHYD (p*EFG1*)	*EFG1* fused with the HA[a] tag under the control of the *PCK1* promoter	35
p99	pUC18 containing *PMT6*	S. Scherer
pCT30	*URA3*-marked *CaARS* vector containing *PMT1*	41
pCT34, pCT35	*URA3*-marked *CaARS* vectors containing *PMT6*	This work
pCT17	As p99, but without the part of the MCS[b] between *Sac*I and *Sph*I	This work
pCT25	Plasmid carrying *pmt6*Δ::*hisG-URA3-hisG* disruption fragment	This work

[a] HA, hemagglutinin.
[b] MCS, multiple cloning site.

(34). Hyphae were induced on solid "Spider" medium (22) or in liquid using serum (9) or 2.5 mM GlcNAc (18) as the inducer.

Sequencing of *PMT6* and plasmid constructions. A plasmid containing *PMT6* was identified in the *C. albicans* genome project (p99) (S. Scherer, personal communication; http://www-sequence.stanford.edu/group/candida). Subfragments of p99 were ligated into pUC19 and sequenced from both ends using M13 forward (U-40) and reverse primers or by using insert-specific oligonucleotides. The 5.3-kb *Hin*dIII fragment containing *PMT6* of p99 was inserted into the *Hin*dIII site of pRC18 (36) to generate replicating plasmids (pCT34 and pCT35 with inverse insert orientation).

Disruption of *PMT6*. For disruption of the *C. albicans PMT6* gene, plasmid pCT17 was cut with *Asp*718 and *Pst*I and the 4.0-kb "Ura blaster" fragment (cut with *Asp*718 and *Pst*I) from p5921 (17) was ligated into this vector. From the resulting plasmid, pCT25, a 5.7-kb *Xho*I fragment (Fig. 1A) was isolated and used for transformation of strains CAI4 and CAP1-3121. Correct insertion of this fragment into one of the two *PMT6* alleles was verified by Southern blotting of DNA of transformants, which was cut with *Bgl*II and *Hin*dIII and probed with a 1.1-kb *Nco*I-*Sal*I fragment derived from the *PMT6* promoter region (Fig. 1A). One of the strains generated, e.g., CAP2-2, with the genotype *pmt6*Δ::*hisG-URA3-hisG*/*PMT6*, was plated out on medium containing 0.02% 5-fluoroorotic acid (5-FOA) (26). Spontaneous 5-FOA-resistant strains were analyzed for loss of the *URA3* sequence by Southern blotting. One of the identified strains, CAP2-23, with the genotype *pmt6*Δ::*hisG*/*PMT6*, was used for a second round of gene disruption with the *C. albicans pmt6* URA blaster fragment of pCT25. Several transformants had the genotype *pmt6*Δ::*hisG*/*pmt6*Δ::*hisG-URA3-hisG*, and strain CAP2-239 was chosen to identify strains by 5-FOA resistance. Strain CAP2-2391 is a representative of mutant strains with the genotype *pmt6*Δ::*hisG*/*pmt6*Δ::*hisG*. The *PMT6* gene was reintroduced into CAP2-2391 by transforming this strain with either plasmid pCT34 or pCT35 (Table 1).

Adherence to endothelial cells. Porcine aortic endothelial cells (PAEC) were isolated from aortas of freshly slaughtered pigs, which were obtained from the local slaughterhouse. The lumina of the aortas were washed with phosphate-buffered saline (PBS; 140 mM NaCl, 4 mM KCl, 1 mM $Na_2HPO_4 \cdot 2H_2O$, 1 mM KH_2PO_4, 12 mM glucose, pH 7.4) under sterile conditions, and the adventitia was removed (43). The remaining part was immersed completely in 30 ml of dispase solution (0.5 mg/ml; Boehringer, Mannheim, Germany) for 15 min at 37°C in an incubator. Afterwards the aortas were fixed on an aluminum tray and the endothelial cells (EC) were scraped off with a rubber policeman. The cells harvested by each scrape were plated in different wells of a six-well dish (pretreated with 0.2% gelatin solution for 30 min at room temperature). PAEC were cultured at 37°C in the humidified atmosphere of an incubator at 5% CO_2–95% air in M199 (Sigma, Munich, Germany) containing 10% fetal calf serum, 30 mM HEPES, 3 mM glutamine, 0.38 g of Dulbecco's PBS/liter, and 4 mg of penicillin and streptomycin/liter. Subcultures were carried out by detaching the cells with trypsin solution (0.5 mg/ml; Sigma), spinning them down at 100 × *g* for 10 min, and plating them in a dilution of 1:7 (approximately 15,000 cells/cm^2). Cells were identified as EC by their cobblestone morphology, the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbo-cyanine perchlorate-labeled acetylated low-density lipoprotein (Paesel, Frankfurt, Germany), and the immunostaining of factor VIII-related antigen.

Monolayers established in six-well plates were used 2 to 4 days after confluency for adhesion assays. The adhesion of *C. albicans* cells was determined as described previously (41).

Animal experiments. Virulence studies were performed as described previously (6). Briefly, strains were harvested from stationary cultures after growth in selective media, adjusted to the desired density in PBS, and injected intravenously into the tail vein in a final volume of 200 μl. The viability of the inoculum was controlled by serial plating of the cells, and the actual infectious load was adjusted accordingly after overnight storage of cells at 4°C. At the time of inoculation viable cell counts were checked again by plating to verify identical loads of infection. After infection, animals were examined for behavioral changes and changes of habit. Survival and mortality were monitored twice a day. Kaplan-Meyer survival graphs were plotted using the GraphPad Prism software, which was also used for log rank test curve comparisons (Mantel-Haenszel test).

Other methods. For RNA preparation cells were grown in YPD to an optical density at 600 nm between 1.3 and 1.9 or they were induced in 2.5 mM GlcNAc as described by Holmes and Shepherd (18). Total RNA was prepared as described by Schmitt et al. (33). RNA blotting was performed as described previously using the 1.5-kb *Cla*I-*Sal*I *ACT1* fragment (9), a 1.5-kb *Bam*HI-*Hin*cII fragment carrying a portion of the *PMT1* coding region (41), and the 1.5-kb *Pst*I-*Xho*I fragment of pCT17 carrying *PMT6* as the probes.

The assay for enzymatic Pmt activity was performed as described previously (41). Although measurements of Pmt activity were highly reproducible, the level of residual Pmt activity remaining in *pmt1*/*pmt1* mutants was found to be variable and to depend strongly on the preparations of Dol-P-[^{14}C]Man and of the acceptor peptide.

Nucleotide sequence accession number. *PMT6* was assigned accession no. AF104916 (GenBank/EMBL).

RESULTS

Sequence of *PMT6*. A clone containing the whole *PMT6* gene (p99) was identified in the *C. albicans* sequencing project (http://www-sequence.stanford.edu/group/candida). The sequence of *PMT6* was determined using M13 standard primers and sequence-specific oligonucleotides. It comprises an open reading frame of 826 codons for a protein with a calculated molecular mass of 94 kDa. Two serine residues at positions 38 and 538 are encoded by nonstandard CUG codons (31). The deduced Pmt6 protein and the *S. cerevisiae* Pmt6 protein have 42% identical residues, whereas Pmt6p is only 21% identical to Pmt1p (41). The identities to other *S. cerevisiae* Pmt proteins were much lower; for this reason the gene was designated *PMT6*. Recently, gene fragments encoding a conceptual Pmt4p homologue of *C. albicans* were also identified in the *C. albicans* sequencing project. Sequence comparisons indicated that Pmt6p is clearly different from Pmt4p (22% identity). Computer analysis predicted that Pmt6p is an integral membrane protein, possibly containing 10 transmembrane domains; 7 of these domains coincide with transmembrane regions that have been determined experimentally in Pmt1p of *S. cerevisiae* (39). Assuming a similar overall structure, the highest degree of identity is present between transmembrane regions I and II and V and VI, respectively, which are the regions constituting both large luminal loops. Pmt6p is also predicted to contain a leucine zipper domain starting at position 723. Four asparagine residues represent potential N glycosylation sites at positions 20, 59, 357, and 453.

Disruption of *PMT6* alleles. The Ura blaster technique (11) was used to disrupt both alleles of *PMT6* in the *C. albicans PMT1*/*PMT1* strain CAI4 and the *pmt1*/*pmt1* mutant strain CAP1-3121 (Table 1; Fig. 1A). Chromosomal DNA of transformants was digested with *Bgl*II and *Hin*dIII and analyzed by Southern hybridization using a specific probe for the *PMT6* promoter. The wild-type *PMT6* allele displayed a 4.9-kb band (Fig. 1B and C, lane 1), whereas a 7.1-kb band was observed in transformants with the Ura blaster integrated into one allele of *PMT6* (Fig. 1B and C, lane 2). After selection on FOA medium, the loss of the *URA3* gene and one copy of the *hisG* element resulted in a 4.3-kb band (Fig. 1B and C, lane 3). The remaining intact *PMT6* allele was disrupted similarly, leading to homozygous *pmt6*::*hisG*/*pmt6*Δ::*hisG-URA3-hisG* strains (Fig. 1B and C, lane 4) and corresponding Ura$^-$ derivatives (Fig. 1B and C, lane 5). Thus, these procedures generated strains lacking only *PMT6* alleles or doubly mutated strains lacking both *PMT6* and *PMT1* alleles. Phenotypes reported below were observed in at least two disrupted or reconstituted strains, which were independently isolated.

Supersensitivity of *pmt6* mutants. We previously observed that homozygous *pmt1* mutants showed increased sensitivities to various antifungals (G418, hygromycin B, and clotrimazole), calcofluor white, and sodium dodecyl sulfate (SDS) (41). For that reason we also tested the susceptibilities of heterozygous and homozygous *pmt6* mutants (Fig. 2A), as well as those of double mutants carrying *pmt1* and *pmt6* disruptions (Fig. 2B), to various agents.

Strains carrying one or two *pmt6* mutant alleles grew well in the presence of 100 μg of hygromycin B/ml, whereas they failed to grow at 200 μg/ml (Fig. 2A). Remarkably, the heterozygous *PMT6*/*pmt6* and the homozygous *pmt6*/*pmt6* mutant showed the same phenotypes. No increased sensitivities to nystatin (10 to 15 μg/ml), amphotericin B (0.5 to 1.5 μg/ml), clotrimazole (1 to 2 μg/ml), fluconazole (5 μg/ml), fluphenazine (50 μg/ml), SDS (0.06%), calcofluor white (10 to 25 μg/ml), G418 (0.8 to 1.2 mg/ml), and sodium orthovanadate (10 to 20 mM) were

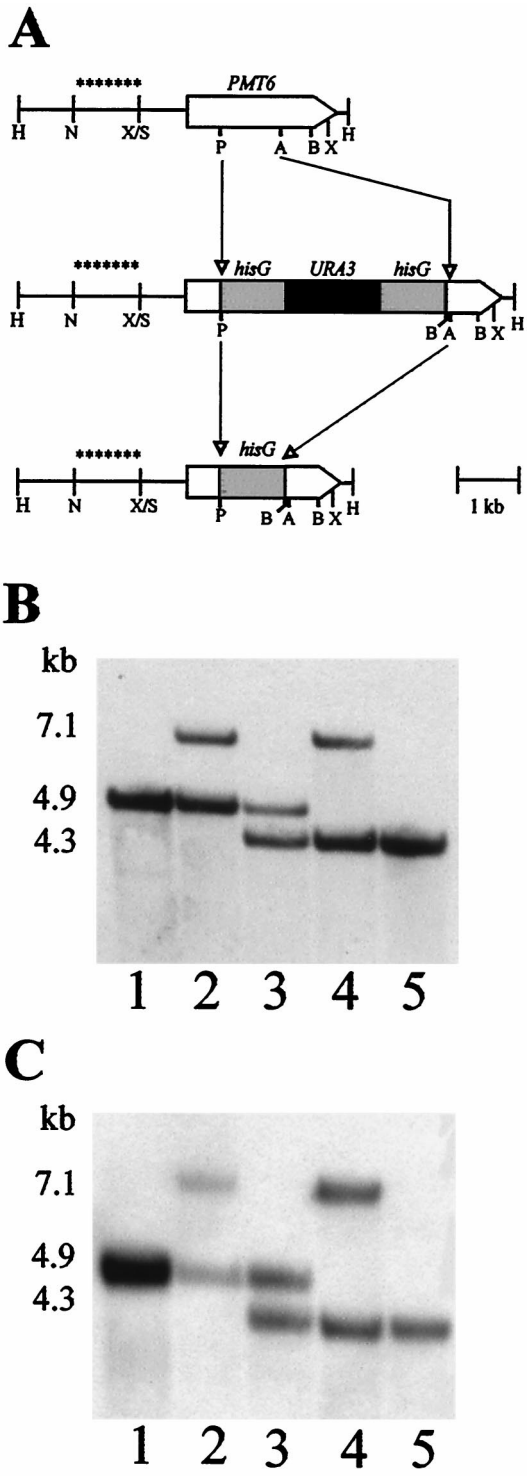

FIG. 1. Sequential disruption of *PMT6* alleles. (A) Schematic representation of the construction of the different alleles. The wild-type *PMT6* gene and the *PMT6* alleles disrupted by the *hisG-URA3-hisG* cassette or by *hisG* are shown. H, *Hin*dIII; N, *Nco*I; X, *Xho*I, S, *Sal*I; P, *Pst*I; B, *Bgl*II; A, *Asp*718. The fragment marked by asterisks was used as a probe for Southern analysis. (B and C) Southern blots of *Hin*dIII-*Bgl*II-digested chromosomal DNA of the following strains: SC5314 (*PMT6*/*PMT6*; lane 1 [B and C]); CAP2-2 (*PMT6*/*pmt6*Δ::*hisG-URA3-hisG*; lane 2 [B]); CAP2-23 (*PMT6*/*pmt6*Δ::*hisG*; lane 3 [B]); CAP2-239 (*pmt6*Δ::*hisG-URA3-hisG*/*pmt6*Δ::*hisG*; lane 4 [B]); CAP2-2391 (*pmt6*Δ::*hisG*/*pmt6*Δ::*hisG*; lane 5 [B]); CPP1 (*PMT6*/*pmt6*Δ::*hisG-URA3-hisG*; lane 2 [C]); CPP11 (*PMT6*/*pmt6*Δ::*hisG*; lane 3 [C]); CPP117 (*pmt6*Δ::*hisG-URA3-hisG*/*pmt6*Δ::*hisG*; lane 4 [C]); CPP1171 (*pmt6*Δ::*hisG*/*pmt6*Δ::*hisG*; lane 5 [C]).

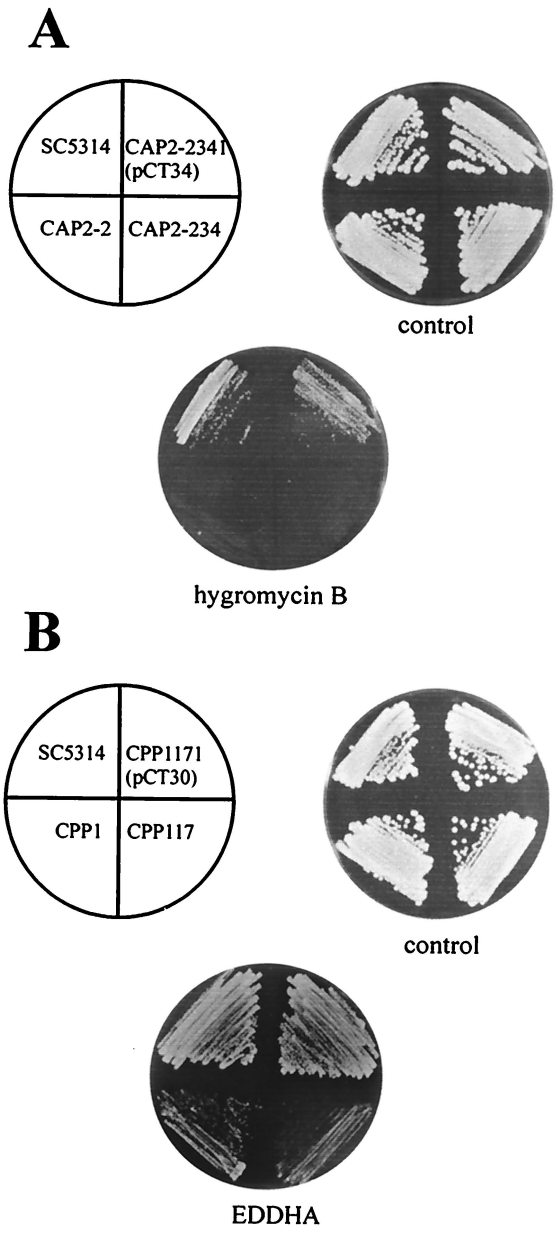

FIG. 2. Sensitivities of *C. albicans* strains. The wild-type strain SC5314 (*PMT1*/*PMT1 PMT6*/*PMT6*) was compared with strain CAP2-2 (*PMT1*/*PMT1 PMT6*/*pmt6*), strains CAP2-234 and CAP2-2341 (*PMT1*/*PMT1 pmt6*/*pmt6*), strain CPP1 (*pmt1*/*pmt1 PMT6*/*pmt6*), and strains CPP117 and CPP1171 (*pmt1*/*pmt1 pmt6*/*pmt6*). Plasmid pCT34 carries *PMT6*, and plasmid pCT30 carries *PMT1*. Strains were grown on YPD medium without or with hygromycin B (200 μg/ml) or on SD medium without or with EDDHA (300 μM). The plates were incubated for 2 days at 30°C.

detected in *pmt6* disruptants. *C. albicans pmt1*/*pmt1* strains with disruptions in at least one *PMT6* allele showed the same phenotype with regard to antifungals as *pmt1* mutants: supersensitivity to G418, SDS, calcofluor white, clotrimazole, and low concentrations of hygromycin B (41). Furthermore, we could detect an increased sensitivity of *pmt1* or *pmt1 pmt6* mutants to Congo red (200 μg/ml), which was not observed in *pmt6* disruptants (data not shown).

Interestingly, the *pmt1 pmt6* mutants also showed new phenotypes that were not detected in strains carrying homozygous single mutations. *pmt1* and *pmt6* single disruptants were resistant to a 300 μM concentration of the iron chelating agent ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA), as was the wild-type strain (SC5314). However, the *pmt1*/*pmt1 pmt6*/*pmt6* double mutant (CPP117) and the *pmt1*/*pmt1 PMT6*/*pmt6* heterozygous strain (CPP1) were no longer able to grow in the presence of 300 μM EDDHA (Fig. 2B). This new phenotype was completely suppressed by overexpression of *PMT1* (plasmid pCT30). In addition to EDDHA sensitivity, slightly reduced growth of strains CPP1 and CPP117 compared to that of singly mutated strains was also observed on YPD medium containing 20 mM caffeine (data not shown).

***PMT6* is required for hyphal morphogenesis.** Hypha formation is induced in certain media including Spider medium (22) or in the presence of positive stimuli including serum or *N*-acetylglucosamine (GlcNAc) (5). We reported previously that *pmt1* mutants are unable to develop hyphae on Spider medium, while they still form hyphae if induced by serum or GlcNAc (41). Therefore, the ability of *pmt6* mutants to form hyphae was tested.

The heterozygous *PMT6*/*pmt6* strains, as well as the homozygous *pmt6*/*pmt6* disruptants showed normal hyphal formation during induction by serum and GlcNAc (data not shown) but a decreased ability to form hyphae on Spider medium (Fig. 3A). Again, as for hygromycin B sensitivity (Fig. 2A), the heterozygous and the homozygous *pmt6* mutants showed identical defective phenotypes. A doubly mutated strain lacking both *PMT1* and *PMT6* alleles was completely morphogenesis negative on Spider medium (Fig. 3B), as were *pmt1* single mutants (41). Reintroduction of *PMT6* into the *pmt6*/*pmt6* mutant restored morphogenesis as expected; in addition, *PMT1* expression complemented the *pmt6* mutant phenotype, suggesting common functions of Pmt6p and Pmt1p. Pmt6p may have a more narrow substrate specificity than Pmt1p since *PMT6* overexpression did not complement the *pmt1* phenotype (Fig. 3A).

Because strains lacking one or two *PMT6* alleles had the same defective phenotype, we speculated that only one allele of *PMT6* was functional, as in the natural heterozygosity of some *C. albicans* strains (32), or that pairing of alleles was required for expression (1). Alternatively, a threshold level of Pmt6p was presumed to be necessary for cells to generate the wild-type morphogenetic phenotype. To determine if *PMT6* is expressed in an allele-specific manner, Northern analyses were performed. All four independent heterozygous *PMT6*/*pmt6* transformants contained a 3-kb *PMT6* transcript (data not shown), suggesting that *PMT6* is not expressed in such a manner.

Because of the effects of *PMT6* on morphogenesis, we tested if *PMT6* expression depends on hyphal induction. For this experiment the *PMT* strain CAI4(pRC2312) was induced by 2.5 mM GlcNAc according to standard procedures (9); at different time points after induction the percentage of hyphae was determined and RNA was isolated for Northern analysis (Fig. 4). The results demonstrate that the *PMT6* transcript level is lowered slightly during the first 50 min of induction, but decreases severalfold after this time; thus, hyphal morphogenesis and *PMT6* transcript levels are not closely correlated. Reprobing the same Northern blot with *PMT1* revealed that the *PMT1* transcript is also not correlated closely to the degree of hyphal development, since it remains relatively constant during the first 50 min of induction and increases only slightly after this time. Figure 4 allows a rough estimation of the relative amounts of both *PMT* transcripts, because probes of similar lengths and specific activities were used. The autoradiographic exposure times were 4 days for *PMT1* and 9 days for

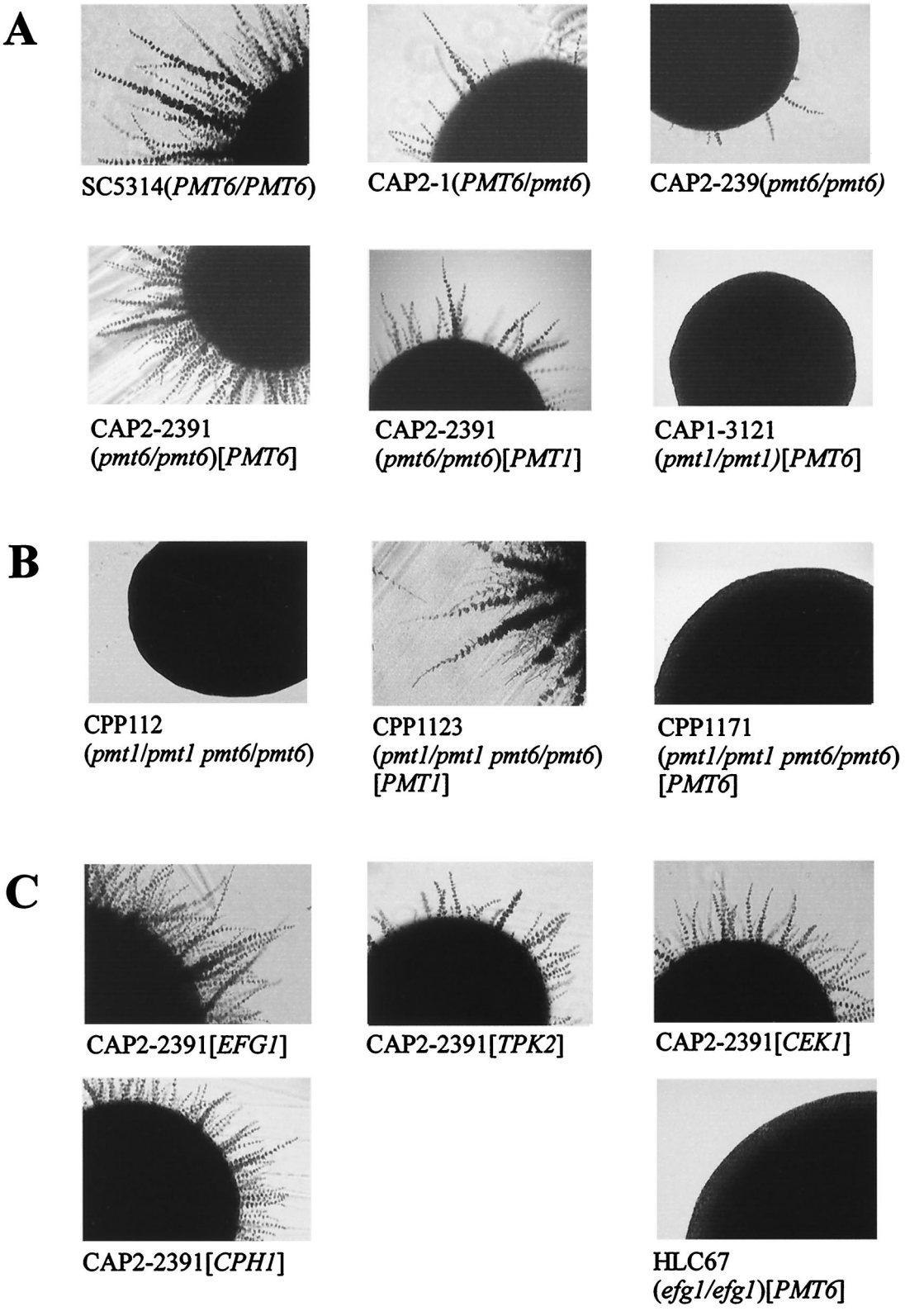

FIG. 3. Hypha formation of *C. albicans* strains. Shown are sections of colonies grown for 3 days on Spider medium at 37°C. (A) Phenotypes of *pmt* single mutants. The indicated *pmt6*/*pmt6* strains were complemented with a plasmid carrying *PMT6* (pCT34) or *PMT1* (pCT30); as controls a *pmt1*/*pmt1* strain complemented by *PMT6* (pCT35) and a wild-type strain (SC5314) were analyzed. (B) Phenotypes of *pmt* double mutants. The indicated *pmt1*/*pmt1 pmt6*/*pmt6* double mutants were transformed with plasmid pCT30 (*PMT1*) or pCT35 (*PMT6*). (C) Suppression of the *pmt6* phenotype by genes encoding signaling components. Strain CAP2-2391 (*pmt6*/*pmt6*) was transformed with plasmids carrying *EFG1*, *CEK1*, *CPH1*, or *TPK2* genes (Table 1). As a control, an *efg1*/*efg1* mutant strain transformed with pCT35 (*PMT6*) was analyzed.

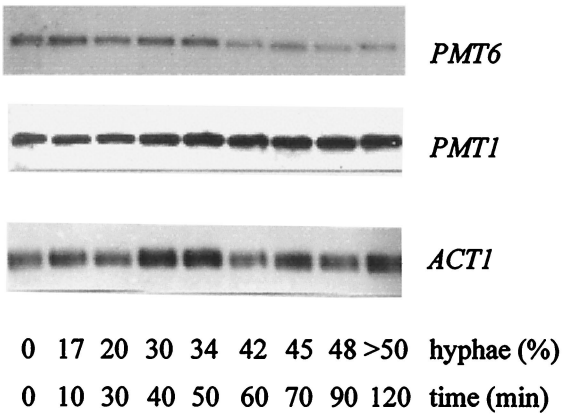

FIG. 4. *PMT6* and *PMT1* transcripts during hyphal induction. The wild-type strain CAI4(pRC2312) was induced to form hyphae in the presence of 2.5 mM GlcNAc. At the indicated times the percentages of hypha-forming cells were determined and total RNA was prepared and analyzed by Northern blotting using probes for the indicated genes.

PMT6. Considering that the *PMT1* signal has about twice the strength of that of *PMT6* at 0 min, we estimate that the level of the *PMT6* transcript is about 25% relative to the level of the *PMT1* transcript; this ratio changes during hyphal formation further in favor of the *PMT1* transcript. The downregulation of the *PMT6* transcript had a different kinetics from that of the downregulation of the *EFG1* transcript described previously (36), because the latter transcript disappeared much more rapidly during hyphal induction. Furthermore, we could show by Northern blottings that high and low *EFG1* expression levels in strain SS4 grown in different media (36) did not alter *PMT6* transcript levels, thus arguing against a close expressional correlation of both genes (data not shown).

Suppression of *pmt6* morphogenetic phenotypes. Conceivably, the inability of *pmt6*/*pmt6* and *PMT6*/*pmt6* strains to form hyphae was due to one of several possibilities, including (i) defects in structural components required for hyphal morphogenesis and (ii) defects in a component of signaling pathways that translate environmental stimuli into alterations of the cell form (e.g., an O-glycosylated membrane "sensor"). Because in *pmt6* mutants hyphal morphogenesis occurred in the presence of serum or GlcNAc, the former hypothesis appeared unlikely. The alternative hypothesis predicts that stimulation of components situated downstream of Pmt6p targets would suppress the morphogenetic defects of *pmt6* mutants. To test this possibility, we transformed the *pmt6*/*pmt6* strain CAP2-2391 with plasmids allowing overexpression of the mitogen-activated protein (MAP) kinase Cek1p and its downstream transcription factor, Cph1p (7, 22), or with plasmids allowing overexpression of the catalytic subunit of the protein kinase A isoform (Tpk2p) (35) and of the morphogenetic regulator Efg1p (36).

As shown in Fig. 3C overproduction of all tested components of signaling pathways was able to relieve the morphogenetic block of the *pmt6*/*pmt6* mutant, although to various degrees. Overexpression of genes encoding transcription factors Efg1p and Cph1p showed a strong complementation, similar to complementation by *PMT6* itself, while overexpression of the genes encoding the kinases Cek1p and Tpk2p resulted in weaker restoration. The different degrees of suppression may be related to promoter strengths, because in the plasmids used *EFG1* and *CPH1* are controlled by the strong *PCK1* and *ADH1* promoters, while the *CEK1* and *TPK2* genes are transcribed by their natural promoters. On the other hand, the complete block of hyphal formation in a *pmt1*/*pmt1* mutant or the partial block of morphogenesis in a *PMT1*/*pmt1* mutant could not be altered by overexpression of the above signaling components (data not shown), suggesting (i) that *pmt1* mutants contain defects unrelated to signaling and (ii) that residual morphogenesis was not responsible for suppression of the *pmt6* morphogenetic defect. We point out that the antifungal sensitivities of the *pmt6* mutants described above could not be suppressed by any of the signaling components (data not shown), indicating that the functions of Pmt6p in morphogenesis and antifungal sensitivities are separable. These results are compatible with the hypothesis that the *pmt6* morphogenetic phenotype is caused by defects in one or more specific components which function upstream of known signaling components, leading to hyphal morphogenesis; however, more-complicated mechanisms cannot be excluded.

TABLE 2. Pmt enzymatic activity in strains

C. albicans strain	Genotype	Amt of [^{14}C]mannose transferred (cpm/min/mg of protein)	% Activity
SC5314	*PMT1*/*PMT1 PMT6*/*PMT6*	7,870 ± 116	100
CAP1-312	*pmt1*/*pmt1 PMT6*/*PMT6*	2,100 ± 206	27
CAP2-239	*PMT1*/*PMT1 pmt6*/*pmt6*	10,170 ± 223	129
CPP117	*pmt1*/*pmt1 pmt6*/*pmt6*	2,000 ± 302	26

Other phenotypes of *pmt6* and *pmt6 pmt1* mutants. The heterozygous *PMT6*/*pmt6* mutant CAP2-2 and the homozygous *pmt6* disruptant CAP2-234 showed the same generation times in SD medium as the wild-type SC5314 or the *PMT6*-reconstituted strain CAP2-2341(pCT34). Furthermore, the homozygous *pmt6*/*pmt6* mutant strains did not aggregate, unlike the *pmt1*/*pmt1* mutants (41), and grew as regular yeast cells. On the other hand, doubly mutated strains lacking both *PMT6* and *PMT1* (strain CPP117) essentially showed the phenotypes of *pmt1* mutants, including slower growth, aggregation, antifungal sensitivity, and complete loss of hyphal morphogenesis on Spider medium (but retained the ability for hyphal morphogenesis in serum media) (41); a few additional sensitivities not present in strains containing single mutations were also observed (see above).

To test if known secreted proteins are modified by Pmt6p, we compared the electrophoretic mobilities in SDS-polyacrylamide gel electrophoresis of several known secreted proteins in *pmt6*/*pmt6* mutants and wild-type cells. Immunoblottings were performed using antibodies to Als1p (19) Int1p (14), Cdr1p (30), and Pma1p (27), as described previously (41). None of the tested proteins showed a different electrophoretic migration in *pmt6*/*pmt6* mutants, indicating that these proteins are not extensively modified by Pmt6p. Furthermore, we did not observe any different activities and intra- or extracellular

TABLE 3. Adhesion of *C. albicans* strains to PAEC

Strain (genotype)	Adhesion (%)[a]
SC5314 (*PMT6*/*PMT6*)	34.6 ± 3.3
CAP2-2 (*PMT6*/*pmt6*)	34.9 ± 3.9
CAP2-3 (*PMT6*/*pmt6*)	39.0 ± 6.9
CAP2-234 (*pmt6*/*pmt6*)	13.1 ± 0.9
CAP2-239 (*pmt6*/*pmt6*)	24.8 ± 6.9
CAP2391(pCT34) (*pmt6*/*pmt6* [*PMT6*])	41.6 ± 5.0

[a] The percentages of 200 *C. albicans* cells adhering to a monolayer of PAEC in 45 min were determined. Values represent the means of three independent measurements ± standard deviations.

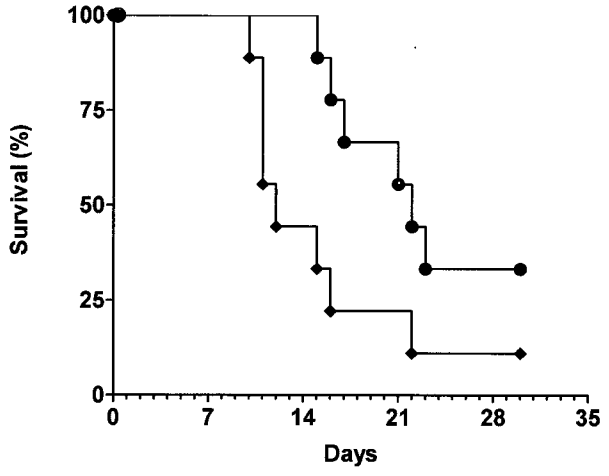

FIG. 5. Virulence of *C. albicans* strains. Strains CAP2-239 (●; *pmt6*/*pmt6*) and the *PMT6*-reconstituted strain CAP2-2391(pCT35) (◆; *pmt6*/*pmt6* [*PMT6*]) were compared. The survival of mice ($n = 12$) injected with 10^5 *C. albicans* cells in the tail vein was determined.

distributions of chitinase activities in *pmt6* mutants compared to those in *PMT6* strains. Different results were obtained previously for *pmt1* mutants, which showed altered migration of Als1p and altered activities and distributions of chitinase (41).

An analysis of the enzymatic Pmt activity was performed by an in vitro assay measuring the transfer of [^{14}C]mannose residues from Dol-P-[^{14}C]mannose to the acceptor peptide acetyl-YATAV-NH_2 (40–42). Wild-type strain SC5314 showed high levels of Pmt activity, while in the homozygous *pmt1* mutants the enzymatic activity was decreased to 27% of wild-type activity (Table 2). In a homozygous *pmt1 pmt6* knockout mutant the Pmt activity was nearly identical (26% compared to that of the homozygous *pmt1* disruptant). Thus, Pmt6p is not detectably active under standard assay conditions and contributes little to the overall Pmt activity of cells. Interestingly, we observed that in a strain with only *PMT6* deleted Pmt activity was increased, rising to 129% of wild-type activity (Table 2). Possibly, in strains lacking Pmt6p a compensatory increase in activities of Pmt1p and/or other Pmt proteins occurs.

Pmt6p is required for adherence and virulence of *C. albicans*. Mannoproteins are necessary for adhesion of *C. albicans* to a number of surfaces (13). Recently we demonstrated that *PMT1* is required for adhesion of *C. albicans* to EC (41). To explore the role of Pmt6p in *C. albicans* adhesion, we tested the ability of heterozygous and homozygous *pmt6* strains to adhere to PAEC. In comparison to wild-type cells (strain SC5314), strains bearing disruptions in both *C. albicans PMT6* alleles adhered less to a monolayer of PAEC (Table 3). While 35% of wild-type cells and 35 to 39% of heterozygous *PMT6*/*pmt6* mutants adhered to the PAEC monolayer in 45 min, the adherence of homozygous *pmt6* disruptants CAP2-234 and CAP2-239 was reduced to 13 and 25%, respectively. Reintroduction of the *PMT6* gene into one *pmt6*/*pmt6* mutant increased adherence, as expected.

To test whether Pmt6p influences the virulence of *C. albicans* in a mouse model of systemic infection, 10^5 cells of the homozygous disruptant CAP2-239 and the reconstituted strain CAP2-2391[pCT35] were injected into the tail vein of immunocompetent mice. The mice were observed for 30 days. The data of Fig. 5 are representative of a total of four independent infections with similar results. Mice infected with the reconstituted wild-type strain (*pmt6*/*pmt6* [*PMT6*]) showed a mean survival time (MST) of 12 days (Fig. 5), while animals treated with the homozygous *pmt6*-disrupted strain survived significantly longer (MST, 22 days). Thus, we could prove that *PMT6* is involved in the virulence of *C. albicans*.

DISCUSSION

The *C. albicans* Pmt6 protein corresponds to one subclass of Pmt proteins in *S. cerevisiae* comprising the Pmt2, Pmt3, and Pmt6 proteins. We previously described the *C. albicans* Pmt1 protein, which corresponds to a second subclass comprising the *S. cerevisiae* Pmt1 and Pmt5 proteins (41). By nonstringent hybridization using *S. cerevisiae PMT1* to *PMT7* probes we only detected two *PMT* genes in *C. albicans* DNA by Southern blotting (41); these genes correspond to *PMT1* and *PMT6*, which are described here. In the *C. albicans* genome sequencing project (http://www-sequence.stanford.edu/group/candida/), which at present covers approximately 95% of the genome (S. Scherer, personal communication), gene fragments designated *PMT1* to *PMT5* have been discovered. Computer comparisons revealed that the genes designated *PMT1* and *PMT5* correspond to the *PMT1* sequence described by us (41), while the gene fragments designated *PMT2* and *PMT3* are identical to *PMT6*, which is described here. Recently, other gene fragments, named *PMT4*, were identified by the *C. albicans* genome project; *PMT4* is different from *PMT1* and *PMT6*. Thus, the present evidence suggests that the set of *PMT* genes in *C. albicans* comprises *PMT1*, *PMT4*, and *PMT6*. The existence of only three *PMT* genes in *C. albicans* differs from what is found for *S. cerevisiae*, which harbors seven paralogous *PMT* genes (38). Deletion of three or four of the seven *PMT* genes is lethal in *S. cerevisiae* (15), while we show here that in *C. albicans* two of the three *PMT* genes can be deleted without a loss in viability. This finding raises the intriguing question of whether proteins other than Pmt proteins could mediate O glycosylation in fungi.

The structure of Pmt6p corresponds to that of the *S. cerevisiae* Pmt proteins, which presumably includes a seven-transmembrane helical configuration in endoplasmic reticulum membranes (39). Nevertheless, although Northern blotting demonstrated that *PMT6* is expressed (although at a lower level than *PMT1*), no effect of *PMT6* deletion on in vitro Pmt enzymatic activity was observed. It is possible that the standard acceptor peptide used in the enzymatic assay does not correspond to the substrate specificity of Pmt6p or that assay conditions able to detect Pmt1p activity fail to reveal Pmt6p. Several of our findings support the hypothesis that Pmt6p does not have a general role in O glycosylation but rather modifies and/or regulates a relatively narrow set of target proteins. First, in vitro Pmt enzymatic activities are identical in extracts of the wild-type and *pmt6* disruptants. Second, we did not observe altered electrophoretic migrations of several secreted and cell wall proteins, including Als1p, Int1p, Cdr1p, and Pma1p, as detected by immunoblotting. The Als1 protein was of special interest because its overproduction in *S. cerevisiae* induced adhesion to host cells (12) and because the electrophoretic migration of Als1p was altered in *pmt1* mutants (41). Third, activity and intra- and extracellular distributions of chitinase were not affected by deletion of *PMT6*, while these parameters were altered in *pmt1* mutants (41). Fourth, the generation of new phenotypes in *pmt6 pmt1* double mutants compared to that in *pmt6* and *pmt1* single mutants, i.e., supersensitivity to the iron chelator EDDHA and to caffeine, suggests that Pmt1p and Pmt6p proteins mannosylate an overlapping, but different, set of target proteins. EDDHA sensitivity has not yet been described in connection with alterations in the cell surface

structure of *C. albicans*. In *S. cerevisiae* some combinations of *pmt* mutations are known to cause increased sensitivities to caffeine (15).

An unexpected phenotype of *C. albicans pmt* mutants was their inability to form hyphae in certain inducing conditions, although growth of the yeast form was unaffected. The *pmt1* mutation led to a complete block of morphogenesis, while the *pmt6* mutant, although severely compromised, still formed short hyphal extensions on solid Spider medium. Current models of dimorphism in *C. albicans* comprise two parallel signaling pathways consisting of a protein kinase (Cek1 MAP kinase or Tpk2 protein kinase A [PKA]) and a transcription factor that are regulated by these kinases (Cph1p or Efg1p) (23, 35, 36). We do not favor the hypothesis that lack of O glycosylation led to defects in, e.g., structural components which are needed for hyphal formation, because hyphae developed in both *pmt1* and *pmt6* mutants in the presence of serum or GlcNAc. Instead, we speculate that O-glycosylated components situated functionally upstream of the Cek1 MAP kinase and/or the Tpk2 PKA were compromised in *pmt* mutants, since we could restore filamentation by overexpression of both kinases and the associated transcription factors. Suppression of only the morphogenetic phenotype, not the antifungal sensitivity phenotype, of *pmt6* strains was observed, indicating different functions of Pmt6p in both processes. Hypothetical components upstream of signaling pathways could, for example, be O-glycosylated sensor proteins located in the cytoplasmic membrane that mediate external signals. To our knowledge this is the first report describing suppression of a specific glycosylation defect by an elevated level of signaling components.

Much of the recent interest in *C. albicans* biology is due to the need to develop new and effective antifungals. In this respect it is of interest that *C. albicans* cells lacking Pmt6p were supersensitive to hygromycin B, a phenotype that in *S. cerevisiae* emerges only if at least two *pmt* mutations are combined or in mutants defective in N glycosylation (8). Because sensitivities to other agents were not observed, it appears that the *pmt6* mutation causes less-drastic sensitivity phenotypes than the *pmt1* mutation. The molecular mechanisms by which O or N glycosylation modify sensitivity characteristics of fungi in general and which contribute to the relatively high intrinsic resistance of *C. albicans* to antifungals and other toxic agents remain to be established. The function of Pmt6p in antifungal resistance is not related to its function in morphogenesis, because overexpression of signaling components (see above) did not alter the antifungal sensitivities of pmt6 mutants. Conceivably, agents lowering levels of resistance to antifungals may be of great interest to complement current antifungal therapies. Furthermore, fungal Pmt proteins are potential selective targets for novel antifungals, because the bulk of O glycosylation in mammals occurs via a biosynthetic pathway different from that in fungi. We have shown here that deletion of *pmt6* generates a significant drop in virulence in a system model of mouse infection, while the *pmt1* mutation produces absolute avirulence (41). The reduced virulence observed for *pmt6* mutants is not caused by a general effect on growth but may involve the block in morphogenesis as described above and/or the defective adhesion to endothelial cells.

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